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ABSTRACT:

Human hematopoietic stem cells expressing recombinant DNA constructs encoding a recombinant molecule containing a signal transducing region and an antigenic specificity region results in the generation of T cells with altered specificities.

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(57) Abstract

Human hematopoietic stem cells expressing recombinant DNA constructs encoding a recombinant molecule containing a signal transducing region and an antigenic specificity region results in the generation of T cells with altered specificities.

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Genetically Modified Human Hematopoietic Stem Cells and their Progeny

INTRODUCTION

This application is a continuation-in-part of United States Serial No. 08/116,825.

Technical Field

The field of this invention is expansion of human hematopoietic stem cells and their genetic modification.

Background Art

Mammalian hematopoietic cells are responsible for an extraordinarily diverse range of activities. They are divided into several lineages, including lymphoid, myeloid and erythroid. The lymphoid lineage, comprising B cells and T cells, produces antibodies, regulates cellular immunity, and detects foreign agents such as disease-causing organisms in the blood. The myeloid lineage, which includes monocytes, granulocytes, and megakaryocytes, monitors the blood for foreign bodies, protects against neoplastic cells, scavenges foreign materials, and produces platelets. The erythroid lineage includes red blood cells, which carry oxygen.

Despite the diversity in the morphology, function, and other characteristics of blood cells, it is presently believed that these cells are derived from a single progenitor population termed "stem cells". These rare primitive cells (approximately 0.01% of bone marrow cells) are distinguished by their high proliferative potential and possible self renewal. Stem cells differentiate into multipotent progenitor cells and ultimately into each of the mature hematopoietic lineages. Thus, stem cells are believed to be capable of generating long term hematopoiesis when transplanted into immunocompromised hosts.

The stem cell was originally defined by the capacity to self-renew and to give rise to progeny that are the committed precursors for all hematopoietic lineages. A number of researchers have concluded from their attempts to divide the progenitor cell compartment into stem cell and committed progenitor cells that these compartments constitute a hierarchy or continuum of cell types whose maturation is characterized by decreases in both pluripotentiality and the ability to repopulate the hematopoietic system of serially transplanted animals.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface "markers." Such markers may be either specific to a particular lineage or progenitor cell or may be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker which was previously indicated as being present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1 myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells and AMPB stands for adult mobilized peripheral blood. As used herein both infra, supra and in Table 1, the negative sign or, uppercase negative sign, (-) means that the level of the specified marker is undetectable above Ig isotype controls by FACS analysis, and includes cells with very low expression of the specified marker.

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers			B cell markers			Myelomonocytic			Other							P-gp Activity
	CD2	CD3	CD8	CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
FBM	-	-	-	-	-	-	-	-	-	?	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	-	-	-	+	?	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

The ability to obtain substantially homogeneous human hematopoietic stem cell (hHSC) compositions offers new approaches to bone marrow transplantation. Since there is evidence to suggest that the stem cell will not be malignant in many cases, by isolating hHSCs and restoring the cells to a host after radiation or chemotherapeutic treatment of cancer or other malignancy the host may be rid of cancerous cells. U.S. Patent No. 5,061,620 describes a substantially homogeneous hHSC composition and the manner of obtaining such a composition. See also the references cited therein.

The definitive T cell marker is the T cell antigen receptor (TCR). There are presently two defined types of TCR; TCR-2 is a heterodimer of 2 disulfide-linked transmembrane polypeptides (α and β), TCR-1 is structurally similar but consists of γ and δ polypeptides. The α and β or γ and δ polypeptides form a heterodimer which contains an antigen recognition site. These heterodimers recognize antigen in association with MHC molecules on the surface of antigen-presenting cells. All of these peptides contain a variable region which contributes to the antigen recognition site and a constant region which forms the bulk of the molecule and includes the transmembrane region and cytoplasmic tail. Both receptors are associated with a complex of polypeptides making up the CD3 complex. The CD3 complex is made up of the γ, ζ and ϵ transmembrane polypeptides. The CD3 complex mediates signal transduction when T cells are activated by antigen binding to the TCR.

A variety of these and related molecules have been cloned and expressed in various T cell lines. Kuwana et al. (1987) Biochem. Biophys. Res. Comm. 149:960-968; Gross et al. (1989) Trans. Proc. 21:127-130; Becker et al. (1989) Cell 58:911-921; Gross et al. (1989) Proc. Natl. Acad. Sci. USA 86:10024-10028; and Goverman et al. (1990) Cell 60:929-939. Dick et al. (1991) Blood 78:624-634 describe gene transfer into normal human

hematopoietic cells employing *in vitro* and *in vivo* assays. Several chimeric TCRs have been created and found to be active in targeting T cells to the antigen recognized by the antibody binding site. Eshhar (1993) Proc. Natl. Acad. Sci. USA 90:720-724; and Hwu et al. (1993) J. Exp. Med. 178:361-366.

Approximately 95% of blood T cells express TCR-2 and up to 5% have TCR-1. The TCR-2 bearing cells can be subdivided further into two distinct non-overlapping populations; the T_H subset which is generally $CD4^+$ and the Tc/s subset which is generally $CD8^+$. $CD4^+$ T cells recognize antigens in association with MHC class II molecules, while $CD8^+$ T cells recognize antigens in association with MHC class I molecules.

The $CD4^+$ set can be further divided functionally into: 1) those cells which positively influence the immune response of T cells and B cells, i.e., the helper cell function; and 2) cells inducing suppressor/cytotoxic functions in $CD8^+$ cells, i.e., the suppressor inducer function.

Other monoclonal antibodies and criteria have been used to subdivide the $CD4^+$ set. For example, all $CD4^+$ cells expressing natural killer (NK) cell markers produce the lymphokine interleukin-2 (IL-2), and do not proliferate in response to antigens and mitogens. In fact, recent *in vitro* studies on $CD4^+$ clones in mouse and man have defined two separate populations (T_{H1} and T_{H2}) based on the production of different lymphokines.

$CD8^+$ T cells can also be subdivided by a number of criteria and a variety of monoclonal antibodies into specific functional subsets. For example, cells which recognize antigen in association with MHC molecules and produce IL-2 ($CD28^+$) and cells which do not recognize antigen in association with MHC molecules or produce IL-2 ($CD11b^+$).

$CD3^+/TCR\text{-}1^+$ cells represent a minority of circulating T cells which are also $CD4^-$, $CD8^-$. These cells home in to surface epithelia such as the epidermis and mucosal epithelia and are termed intra-epithelial lymphocytes (IEL). In interstitial mucosal epithelium $TCR\text{-}1^+$ cells also express CD8. It is probable that these cells represent a primitive cytotoxic population operating at the sites of entry of pathogens. CD2 is also found on about 50% of $CD3^-$NK cells. CD5 molecules are expressed on all T cells, and also on a subpopulation of B cells which are involved in autoantibody production.

B lymphocytes represent about 5-15% of the circulating lymphoid pool and are classically defined by the presence of endogenously produced immunoglobulins (antibody). These molecules are inserted into the surface membrane where they act as specific antigen receptors. They are detected on the surface of mature cells by staining cells suspensions with fluorochrome-labelled specific antibodies to the appropriate immunoglobulin of the species under investigation.

The majority of human peripheral blood B lymphocytes express both surface IgM and IgD molecules, which share the same specificity on the same cell. Very few cells express surface IgG, IgA or IgE in the circulation although these are present in larger numbers in specific locations in the body, for example, IgA-bearing cells in the intestinal mucosa.

A number of other markers are carried by both mouse and human B cells but not by resting T cells. The majority of B cells carry MHC class II antigens which are important in cooperation with T cells. Fc receptors for IgG (FcRII, Cdw32) are also present. CD19, CD20 and CD22 are the main markers currently used to identify human B cells.

The literature cited herein is hereby incorporated by reference.

SUMMARY OF THE INVENTION

The present invention provides genetically modified stem cells and lymphoid progenitor cells that express recombinant DNA encoding an antigenic specificity region and a signal transducing region. These recombinant DNAs include recombinant TCRs and chimeric TCRs. The recombinant TCRs are full length α and β chains of known antigen binding specificity. The chimeric TCRs are genetically engineered such that they contain a functional signal transducing region and a functional antibody-derived antigen binding site. T cell specific expression of the recombinant DNA enables the T cell progeny of the transduced stem cells to bind to antigen and exert T cell functions including, but not limited to, cytolysis and B cell helper activity. As described herein, the transduction of stem cells introduces genes encoding the recombinant DNA. The production of T cells with the recombinant DNA has the potential to broaden the immunotherapy of viral infections, including, but not limited to, human immunodeficiency virus (HIV) and hepatitis B, and to extend and specifically target cancer treatment to multiple cancer histologies.

Also included are the genetically modified hHSCs containing the recombinant DNA molecules.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The unique characteristics of stem cells distinguish them from other cells which have been successfully genetically engineered. Efforts to genetically engineer the progeny of stem cells frequently encounter lack of transmission of functional expression of the introduced construct in cells, intermittent expression, and the like. Therefore, even where one has demonstrated the successful integration of the construct in such cells, subsequent growth of the progeny cells and their differentiation may result in the failure of the construct to function.

In addition, by appropriate use of inducible promoters, expression of various protein products can be achieved at selected levels of differentiation or in selected cell lineages, or even in response to particular chemicals, such as chemoattractants, particular ligands, and the like. Also, as there is better understanding of the manner in which stem cells differentiate to specific lineages, particular lineages, such as megakaryocytes, subsets of T cells, monocytes, and the like can be produced in culture.

Currently, T cell gene therapy requires *ex vivo* expansion of T cells with cytokines. Upon re-infusion, the modified T cells often do not home properly to their target organs and may become trapped in (and cleared by) the lungs, liver or spleen. This improper homing may be due to alteration of the membranes during the *ex vivo* processing, downregulation of homing receptors, or the like. *Ex vivo* expansion of T cells is costly, cumbersome and time consuming and thus less than ideal for treatment. As opposed to mature T cells, it would be advantageous to transduce stem cells with TCRs and chimeric TCRs as described herein. Use of such modified stem cells would obviate the necessity of *ex vivo* expansion of the effector T cells and thus concerns of altered trafficking and persistence in vivo.

Since T cells have a finite life span, the process of transduction of mature T cells and infusion must be repeated, whereas stem cells self-renew and, therefore, transduced HSCs are able to differentiate to replenish lost T cells. The recombinant TCR can be put under the control of a T cell specific promoter so that it is only expressed in T cells. For example, the promoter could be Granzyme A, which would cause the recombinant TCR to be expressed predominantly in NK cells and cytotoxic T lymphocytes (CTLs). The T cells derived from a transduced stem cell should home and circulate properly since they have matured *in vivo* and have not been

manipulated subsequently *ex vivo*. They can then be expanded in number by administering cytokines *in vivo*. Since primarily antigen-activated cells proliferate in response to cytokines, modified T cells recognizing the target antigen should be relatively amplified. Also, it may be possible to get a stronger response from the T cells derived from transduced stem cells. If more mature T cells are transduced with the recombinant TCR, they may have a dampened response if they are "memory" cells (i.e. previously exposed to antigens) and, therefore, "biased."

Another advantage to genetically modified HSC over mature T cells would be the ability to express the recombinant TCR in more than one hematopoietic lineage. For example, since macrophages are known to have the ability to engulf tumor cells, it may be useful to express the recombinant TCR in macrophages.

It is also possible to transduce the recombinant TCR into the primitive lymphoid-committed population described in U.S. Patent Application Serial No. 08/260,185, filed June 15, 1994. This is preferable to transducing mature T cells in that the progenitors have some limited regenerative capacity and thus, while still transient, will obviate the need for *ex vivo* T cell expansion and reduce the frequency of administration. Also, gene transfer is likely to be more efficient in progenitors than in stem cells because progenitor cells cycle more actively than stem cells and retroviral vectors are known to require actively cycling cells for efficient integration of the recombinant DNA.

Substantially homogeneous hHSCs can be maintained in long-term cultures and expanded in number in appropriate media, optionally in conjunction with hematopoietic factors such as LIF, stem cell factor, IL3, IL6, IL7, IL11, GCSF, GMC, EPO, MIP-1α and IFNγ, under otherwise conventional conditions. The hHSCs may be maintained in culture for long periods of time, as demonstrated by

their capacity to continually differentiate into multilineage progeny.

Genetic modification of the hHSC can be accomplished at any point during their maintenance by transducing a substantially homogeneous stem cell composition with a recombinant DNA construct described herein. Preferably, a retroviral vector is employed for the introduction of the DNA construct into the hHSC host. The resulting cells may then be grown under conditions similar to those for unmodified hHSC, whereby the modified hHSC may be expanded and used for a variety of purposes.

The hHSC which are employed may be fresh, frozen, or have been subject to prior culture. They may be fetal, neonatal, or adult; and obtained from liver, bone marrow, blood or any other conventional source. The manner in which the stem cells are separated from other cells, whether of the hematopoietic or of other lineage is not critical to this invention. Conveniently, the cells may be separated as described in U.S. Patent No. 5,061,620.

As described, the substantially homogeneous composition of hHSC may be obtained by selective isolation of cells free of markers associated with differentiated cells, while displaying epitopic characteristics associated with stem cells. At such time as a specific marker is identified for hHSC, binding of an antibody to such marker may provide the desired composition.

A large proportion of the differentiated cells may be removed by initially using a relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation may be employed to remove erythrocytes, by employing Ficoll-Hypaque separation.

The gross separation may be achieved using methods known in the art including but not limited to magnetic beads, cytotoxic agents, affinity chromatography or panning. Antibodies which find use include antibodies to lineage specific markers which allow for removal of most, if not all, mature cells, while being absent on hHSC.

Concomitantly or subsequent to a gross separation, which provides for positive selection, a negative selection may be carried out, where antibodies to specific markers present on dedicated cells are employed. For the most part, these markers include, but are not limited to, $CD3^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD14^-$, $CD15^-$, $CD19^-$, $CD20^-$, $CD33^-$ and glycophorin A; preferably including, but not limited to, at least $CD3^-$, $CD8^-$, $CD10^-$, $CD19^-$, $CD20^-$ and $CD33^-$; and normally including at least $CD10^-$, $CD19^-$ and $CD33^-$. See Table 1. The hematopoietic cell composition substantially depleted of dedicated cells may then be further separated using a marker for Thy-1, whereby a substantially homogeneous stem cell population is achieved. Exemplary of this stem cell population is a population which is $CD34^+Thy\text{-}1^+$, which provides an enriched stem cell composition.

An hHSC composition is characterized by being able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and higher order cultures, and being capable of differentiating into the various lymphocytic and myelomonocytic lineages, particularly B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

The hHSC may be grown in culture in an appropriate nutrient medium, including, but not limited to, conditioned medium, a co-culture with an appropriate stromal cell line, adhesion molecules, or a medium comprising a synthetic combination of growth factors

which are sufficient to maintain the growth of hematopoietic cells.

For conditioned media or co-cultures, various stromal cell lines may be used. Since human stromal cell lines are not required, other stromal cell lines may be employed, including but not limited to rodentiae, particularly murine. Suitable murine stromal cell lines include AC3 and AC6, which are described in Whitlock et al. (1987) Cell 48:1009-1021. Other stromal cell lines may be developed, if desired. Preferably, the stromal cell line used is a passage of AC6, AC6.21 (otherwise referred to as SyS1).

Various devices exist for co-culture of hHSC with stromal cells which allow for growth and maintenance of stem cells. These include devices employing mechanisms including but not limited to crossed threads, membranes and controlled medium flow. These may be employed for the growth of the cells for removal of waste products, and replenishment of the various factors associated with cell growth.

Conveniently, tissue culture plates or flasks may be employed where confluent stromal cell layers may be maintained for extended periods of time without passage, but with changing of the tissue culture medium about every five to seven days.

The hHSC may be grown in co-culture by placing the hHSC onto the stromal cell lines, either directly or separated by a porous membrane. For example, about 3 x 10^4 to 3 x 10^5 cells/ml are placed on a confluent stromal cell layer. The media employed in the co-culture may be any convenient growth medium, including but not limited to RPMI-1640 and IMDM either individually or in combination, where appropriate antibiotics to prevent bacterial growth, e.g. penicillin, streptomycin (pen/strep) and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol (1-10 x 10^{-5}

M, 2-ME) and from about 5-15%, preferably about 10% of serum, *e.g.* fetal calf serum (FCS).

In addition to other additives, LIF may be added in from about 1 ng/ml to 100 ng/ml, more usually 5 ng/ml to 30 ng/ml if the cells are to be expanded prior to transduction. Other factors may also be added, including but not limited to, interleukins, colony stimulating factors, steel factor. Of particular interest are LIF, IL-3, IL-6, GM-CSF and MIP-1α.

The factors which are employed may be naturally occurring or synthetic, *e.g.* prepared recombinantly, and may be human or of other species, *e.g.* murine, preferably human. The amount of the other factors will generally be in the range of about 1 ng/ml to 100 ng/ml. Generally, for IL-3, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 100 ng/ml; for IL-6, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml, and for GM-CSF, the concentration will generally be 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml.

In one embodiment, the stem cells are optionally expanded prior to or after transduction. During expansion, the growth factors may be present only during the initial course of the stem cell growth and expansion, usually at least 24 hours, more usually at least about 48 hours to 4 days or may be maintained during the course of the expansion.

For use in clinical settings, it is preferable to transduce the stem cells without prior or subsequent expansion. In one embodiment therefore, the stem cells are cultured with or without LIF or other factors in an appropriate medium, transduced with the appropriate vector, cultured for approximately 72 hours and reintroduced into the host.

For genetic modification of the hHSC, usually a retroviral vector will be employed, however any other suitable vector may be used. These include, but are not

limited to, adenovirus, adenoassociated virus and artificial chromosomes derived from yeast. Combinations of retroviruses and an appropriate packaging line are also suitable, where the capsid proteins will be functional for infecting human cells. Various amphotropic virus-producing cell lines are known, including, but not limited to, PA12 (Miller et al. (1985) Mol. Cell. Biol. 5:431-437); PA317 (Miller et al. (1986) Mol. Cell. Biol. 6:2895-2902); and CRIP (Danos et al. (1988) Proc. Natl. Acad. Sci USA 85:6460-6464).

Possible methods of transduction include, but are not limited to, direct co-culture of hHSC with producer cells e.g. by the method of Bregni et al. (1992) Blood 80:1418-1422 or culturing with viral supernatant alone with or without appropriate growth factors and polycations e.g. by the method of Xu et al. (1994) Exp. Hemat. 22:223-230; and Hughes et al. (1992) J. Clin. Invest. 89:1817.

The constructs employed will normally include a marker gene, which allows for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct. Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin. Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections could be accomplished by employment of a stable cell surface marker to select for transgene expressing hHSC by FACS sorting.

The constructs can be prepared in a variety of conventional ways. Numerous vectors are now available which provide the desired features, such as long terminal repeats, marker genes, and restriction sites, which may be further modified by techniques known in the art. The constructs will encode a signal peptide sequence in

addition to the antigenic specificity region and cytoplasmic signalling sequence, to ensure that the recombinant TCR is properly processed post-translationally and expressed on the cell surface. Preferably, the construct is under the control of a T cell specific promoter. Suitable T cell specific promoters include, but are not limited to, GranzymeA and CD8.

In one embodiment, the signal transducing region and antigenic specificity region are both obtained from TCRs ("classic TCR"). In another embodiment the constructs encode chimeric polypeptides comprising the signal transducing region obtained from a T cell specific receptor or the Fcγ receptor and an antigen binding portion of an immunoglobulin ("chimeric TCR").

The recombinant classic TCRs are functional, preferably full length, TCR α and β or γ and δ polypeptides which have been derived from a T cell with known antigenic specificity. Suitable sources of antigen-specific TCRs include, but are not limited to, cytotoxic T lymphocytes, T helper cells and natural killer cells. In another embodiment, the polypeptides may be recombined so as to form a single functional polypeptide with the Vα and Vβ regions forming the antigen binding site. In another embodiment, the Vα and Vβ regions from different TCRs may be recombined to endow the TCR with a different specificity.

In the case of the chimeric TCR, the chimeric molecule contains an antigen binding sequence from an antibody, a transmembrane sequence and a sequence that can transduce a T cell specific function. Suitable signal transducing regions can be obtained from T cells including, but not limited to, the γ chain of the F_C receptor, the TCR ζ chain, and the IL-2 receptor α, β or γ chains. Suitable antigen binding domains may be derived from antibodies that specifically bind the target antigen. These include, but are not limited to,

antibodies that recognize viruses, bacteria and cancer cells. Preferably, the functional portion of the chimeric molecule is the constant region of a Fcγ or ζ polypeptide and the antigen binding domain is a variable region of an antibody. The variable region may be either the V_H or V_L regions or a single chain recombinant thereof.

The T cell progeny of the stem cells containing the recombinant classic TCR polypeptides are "MHC restricted", that is, they will only recognize antigen in the presence of MHC. Thus, when using these cells to treat a patient, the TCRs must be of the same haplotype of the host. It is well within the skill of one in the art to match the haplotype of the host and the TCR.

The T cell progeny of the stem cells containing the chimeric TCR molecules, will recognize antigen in the absence of MHC and thus will not be MHC restricted. These molecules are suitable for use in all hosts regardless of haplotype.

The hHSC may be genetically modified so that upon reintroduction into the host and subsequent differentiation, T cells are produced that are specifically directed against any antigen. Generally, suitable antigens include, but are not limited to, those found on virally infected cells, and specific cancer cells. More specifically, suitable antigens include, but are not limited to, viral coat proteins and specific surface proteins of cancer cells.

The modification of an hHSC cell population with the DNA constructs described herein endows all progeny T cells with the new specificity. Such modified T cells can be used to treat any disease or cancer to which there is available a specific antibody. Monoclonal antibodies directed against many different tumor antigens are available and can be used to generate tumor-specific chimeric receptors.

In the case where the specificity is for cancer cells, the T cells will home to the cancer and effect T cell activity including but not limited to cytolysis and/or B cell helper activity. These activities will be effective in eradicating cancer cells. In the case where the specificity is for viral proteins, the T cells will home to virally infected cells and effect cell lysis to prevent further replication of the virus. The effect may be potentiated by the systemic co-administration of recombinant cytokines (e.g. IL-2 or IL-3 by either boosting reactivity, function or causing expansion of the relevant cells in situ.

In order to determine if the DNA constructs described herein are functional, a T cell line can be transduced with the constructs and assayed for activity resulting specifically from the genes expressed by the construct. For instance, for testing for activity of a construct encoding a cancer cell specific recombinant polypeptide, the transduced T cell line would be assayed for its ability to lyse cancer cells expressing the protein recognized by the recombinant polypeptide but not cells that do not express this polypeptide. Once activity of the recombinant polypeptide is confirmed, the DNA construct can be used to transduce stem cells. Also included are the stem cells containing the DNA constructs described herein.

The vectors containing the polynucleotides of interest can be introduced into the stem cells by any of a number of appropriate means, including, but not limited to, electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection or transduction (where the vector is an infectious agent, such as a retroviral genome). As used herein, the terms transduction and infection are interchangeable. Although the term "transduction" is often used herein to describe

introduction of the DNA constructs into the stem cells, any other suitable method may be used.

In many situations, cell immunotherapy involves removal of bone marrow or other source of stem cells from a human host, isolating the stem cells from the source and expanding the stem cells. Meanwhile, the host may be treated to partially, substantially or completely ablate native hematopoietic capability. The isolated stem cells may be modified during this period of time, so as to provide for stem cells having the desired genetic modification. After completion of the treatment of the host, the modified stem cells may then be restored to the host to provide for the new capability. The methods of stem cell removal, host ablation and stem cell repopulation are known in the art. If necessary, the process may be repeated to ensure the substantial repopulation of the modified stem cells. Immunotherapies using modified stem cell populations to produce T cells with new specificities circumvent the cumbersome ex vivo process of isolating, expanding and transducing mature T cells.

To ensure that the stem cells have been successfully modified, a vector-specific probe may be used to verify presence of the vector in the transduced stem cells or their progeny. Proper expression of the construct on the cell surface may be detected by, for example, an anti-idiotypic antibody or by assays for T cell activation which will depend on the recombinant TCR binding to the target antigen (e.g., cytokine release). In addition, the cells may be grown under various conditions to ensure that they are capable of maturation to all of the hematopoietic lineages while maintaining the capability, as appropriate, of the introduced DNA. Various tests *in vitro* and *in vivo* may be employed to ensure that the pluripotent capability of the stem cells has been maintained.

For erythrocytes, one may use conventional techniques to identify BFU-E units for example methylcellulose culture. Metcalf, In: Recent Results in Cancer Res. (1977) Vol. 61, Springer-Verlag, Berlin, pp. 1-227. Thus demonstrating that the cells are capable of developing the erythroid lineage.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured for 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in U.S. Patent No. 5,147,784, particularly transplanting under the kidney capsule. After 6-10 weeks, assays of the thymus fragments injected with the cells can be performed and assessed for donor derived T cells.

Further demonstration of the multilineage capacity of the transduced cell populations may be accomplished by the detection of continued myeloid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) Blood 79:1704; and Galy et al. (1994) Blood 84:104. To analyze this, one may isolate human fetal bone and transfer a longitudinally sliced portion of this bone under the skin of a scid/scid animal: the bone cavity is diminished of endogenous cells by whole body irradiation of the mouse host prior to infection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the host bone cells. Stem cells from human hematopoietic sources have been found to sustain B lymphopoiesis and myelopoiesis in the SCID-hu bone model.

The modified stem cells may be administered in any physiologically acceptable vehicle, normally intravascularly, although they may also be introduced into bone or other convenient site where the cells may

find an appropriate site for regeneration and differentiation (e.g., thymus). Usually, at least 1 x 10^5 cells will be administered, preferably 1 x 10^6 or more. The cells may be introduced by injection, catheter, or the like. If desired, factors may also be included, including, but not limited to, interleukins, *e.g.* IL-2, IL-3, IL-6, and IL-11, as well as the other interleukins, the colony stimulating factors, such as G-, M- and GM-CSF, interferons, e.g. γ-interferon, erythropoietin.

The following examples are provided to illustrate but not limit the invention.

Example 1

Genetic Modification of Highly Enriched Stem Cell Population

The vector used was Sp6-γ, described in Eshhar et al. (1993) Proc. Natl. Acad. Sci. USA 90:720-724, which has the variable regions of the anti-TNP antibody Sp6 and the γ chain of the Fc receptor. The vector was transfected into the packaging cell line PA317.

Approximately 200,000 cells of the $CD34^{hi}Lin^-$ phenotype were seeded onto irradiated Sp6-γ producer cells (3K rads). $CD34^{hi}Lin^-$ stem cells were isolated from fetal bone marrow by the method described by DiGiusto et al. (1994) Blood 84:421-432. "Lin^-" refers to cells lacking lineage-specific markers; in this case, Lin^- refers to cells lacking CD2, 14, 15, 16, 19 and glycophorin A. The fetal bone marrow stem cell source was determined to $GAPA^+$.

The $CD34^{hi}Lin^-$ cells were seeded into 2 wells of a 12-well dish containing Sp6-γ producer cells in 2 ml of Whitlock-Witte media containing LIF (50 ng/ml) and IL-6 (10 ng/ml).

The cells were harvested 72 hours later for injections into SCID-hu thymus/liver (Thy/Liv) grafts.

Example 2

In Vivo Test of the Ability of Stem Cells Transduced with a Recombinant Virus Carrying the Chimeric T-Cell Receptor to Yield T-Cells Expressing the Chimeric T-Cells Receptor

CB-17 *scid/scid* (SCID) mice bred in facilities at SyStemix, Inc., Palo Alto, California, were used between 6 to 8 weeks of age for the construction of SCID-hu Thy/Liv mice according to the method described by Namikawa et al. (1990) J. Exp. Med. 172:1055-1063. The human fetal thymus and liver tissue used was GAPA.

Approximately 39,000 transduced cells per graft were injected directly into the Thy/Liv grafts of three SCID-hu Thy/Liv animals (mice 2, 3, 4). The SCID-hu Thy/Liv animals were irradiated with 150 Rads 1-2 hours prior to injection of the transduced cells to partially ablate endogenous stem cells from the fetal liver implant. One animal was not injected with donor cells and was not irradiated (mouse 1). The mouse-1 graft was neither reconstituted nor irradiated, and thus yielded normal human T cell populations which did not contain transduced cells and therefore served as a negative control.

FACS Analysis of Donor Reconstitution and Expression of the Chimeric Receptor in the Grafts:

Four months after inoculation, the animals were sacrificed. The Thy/Liv grafts were removed and a single cell suspension was prepared in PBS, 3% FBS, 10 mM Hepes. The cell suspensions were split in half and processed as follows.

To test whether the donor-derived T cells from the SCID-hu mice expressed the chimeric receptor, TNP was first conjugated to phycoerythrin (TNP-PE) (Clarence Ahlem, SyStemix). Since cells expressing the anti-TNP chimeric receptor should bind the TNP-PE, they should then be detectable by FACS analysis.

One-half of the cells were stained with: (1) CD4-FITC; (2) CD8-TriColor; (3) TNP-PE; propidium iodide was

included in the final suspension for live/dead discrimination.

The other half of the cells were stained with: (1) CD4-PE; (2) CD8-TriColor; (3) GAPA-FITC; propidium iodide was included in the final suspension for live/dead discrimination. The labelled antibodies were obtained from Becton Dickinson. The results obtained are presented in Table 2 where NI stands for not injected and ND stands for not determined due to insufficient cells. GAPA-FITC recognized the donor cells. When total thymocytes from the grafts were incubated with the TNP-PE and analyzed by FACS, a significant number of cells were PE-positive compared to negative controls as shown in Table 2.

TABLE 2

FACS ANALYSES

Mouse	Virus	Percent TNP$^+$ Events			Percent Donor Cells (GAPA$^+$)			
		CD8$^+$	CD4$^+$8$^+$	CD4$^+$	CD8$^+$	CD4$^+$8$^+$	CD4$^+$	TOTAL
1	NI	0.69	0.06	0.19	NA	NA	NA	NA
2	Sp6-γ	2.23	0.61	0.49	14.05	1.28	10.25	5.00
3	Sp6-γ	ND	ND	ND	12.35	3.06	10.90	10.90
4	Sp6-γ	1.63	0.22	0.03	3.50	0.19	3.75	1.20

Example 3

Cell Therapy for HIV-1 Infections

An autologous hHSC population is genetically modified such that differentiated T cells derived from the population express TCRs that enable the cell to recognize and destroy HIV infected cells.

Construction of Vector. A segment of DNA is engineered to encode a chimeric TCR. Eshhar et al. (1993) Proc. Natl. Acad. Sci. USA 90:720-724. The variable portion of the receptor consists of the variable region (Fv) of an immunoglobulin that recognizes peptides of the viral envelope of HIV (e.g. gp160). The signal transduction region consists of either the ζ chain of the TCR/CD3 complex or the γ chain of the Fc receptor (FcR); the TCR α or β chains could also be used as the signal transducers. The Fv portion is expressed as a single chain (scFV), with the V_L domain bridged to the V_H domain of the antibody via a flexible linker. The recombinant

TCR is fused to the SC kappa leader peptide to ensure translocation to the cell membrane according to the method described by Eshhar et al. The resulting recombinant protein has the following structure (amino- to carboxy-terminus): SC kappa leader peptide-V_L-V_H-ζ chain of the TCR, or γ chain of the FcR. TCR α or β chains may also be used as the signaling molecules.

The DNA encoding the receptor is inserted into the LXSN bicistronic retroviral vector according to the method described by Miller and Rosman (1989) BioTechniques 7:980, under the transcriptional control of the LTR from the Moloney Murine Leukemia Virus, along with the neomycin phosphotransferase gene under the transcriptional control of the SV-40 early region promoter. Transcription of the chimeric receptor is under the control of an endogenous T cell promoter. Using this system, is possible to package scFv-ζ and scFv-γ transcripts into retroviral particles and transduce hHSC's, selecting for G418 resistance.

Example 4
Cell Therapy

During modification of the cells, the host is treated to partially, substantially or completely ablate native hematopoietic capability. After completion of the treatment of the host, the modified stem cells are then restored to the host to provide for the recognition and destruction of HIV infected cells. If necessary, the process is repeated to ensure the substantial absence of the original host cells and the substantial population of the modified stem cells. T cells derived from gene-modified hHSC that mature in a patient after the myeloablative regimen are endowed with specificity to HIV-1. To prevent HIV-1 infection of T cells, hHSC is co-transduced with vectors encoding TAR decoys or rev mutants. Gene-modified hHSC is admixed with unmodified

hHSC to endow the patient with a broad T cell repertoire. Patients may then be given recombinant interleukin-2 systemically to expand the HIV-1 reactive T cell population.

Example 5
Cell Therapy for Cancerous Tumors

An autologous hHSC population is genetically modified such that differentiated T cells derived from the population express chimeric TCRs that enable the cell to recognize and destroy specific types of cancer cells.

Construction of Vector. A segment of DNA is engineered to encode a chimeric TCR as described above. In this case, the variable portion of the receptor consists of the variable region (Fv) of an immunoglobulin that recognizes peptides found on the surface of a particular cancer cell type. An example is the monoclonal antibody MOv18 which is specific for human ovarian carcinoma. Hwu et al. (1993) J. Exp. Med. 178:361-366. The signal transduction region consists of either the ζ chain of the TCR or the γ chain of the FcR. The Fv portion is expressed as a single chain (scFV), with the V_L domain bridged to the V_H domain of the antibody via a flexible linker as described by Eshhar et al. The recombinant TCR is fused to the SC κ leader peptide to ensure translocation to the outer cell membrane. The resulting recombinant protein has the following structure (amino- to carboxy-terminus): SC κ leader peptide-V_L-V_H-ζ chain of the TCR, or γ chain of the FcR.

The DNA encoding the receptor is inserted into the LXSN bicistronic retroviral vector as described by Miller and Rosman, under the transcriptional control of the LTR from the Moloney Murine Leukemia Virus, along with the neomycin phosphotransferase gene under the transcriptional control of the SV-40 early region

promoter. Transcription of the chimeric receptor is under the control of an endogenous T cell promoter. Using this system, is possible to package scFv-ζ and scFv-γ transcripts into retroviral particles and transduce hHSC's, selecting for G418 resistance.

Genetic modification of $CD34^+Thy\text{-}1^+$ Population. Bone marrow or mobilized peripheral blood is removed from the human host using standard techniques. $CD34^+Thy\text{-}1^+$ cell populations are isolated and expanded using the techniques outlined above. The infection is performed by introducing approximately 10^4 $CD34^+Thy\text{-}1^+$ cells and 1 x 10^6 CFU of the virus in 1 ml of long-term culture medium ("LTCM") comprising 10 ng/ml of LIF, IL-3, IL-6, and GM-CSF. The cell mixture is maintained for at least 24 hours, and the medium diluted to 10 ml with LTCM and 100 μl of the medium introduced into wells in which confluent layers of the AC6.21 stromal line is present. The cells from 5-15 wells, each of the cells with and without virus, are then introduced into a methylcellulose culture and maintained for 2 weeks. At the end of this time, all cells are collected from each methylcellulose culture and analyzed using Southern and Northern analysis to confirm successful gene insertion and transcriptional expression.

Cell Therapy. During modification of the cells, the host is treated to substantially or completely ablate native hematopoietic capability. After completion of the treatment of the host, the modified stem cells are then restored to the host to provide for the recognition and destruction of specific types of cancer cells. If necessary, the process is repeated to ensure the substantial absence of the original host cells and the substantial population of the modified stem cells. T cells derived from gene-modified hHSC that mature in a patient after myeloablative regimen are endowed with specificity to the carcinoma to which the binding region of the antibody has specificity. In the case of antibody

MOV18, the T cells have specificity for ovarian carcinoma. Further expansion (or activation of) the relevant T cell population could be achieved by the systemic administration to the patient of appropriate recombinant cytokines (e.g. IL-2, IL-7).

Example 6
Cell Therapy for Cancerous Tumors

An autologous hHSC population is genetically modified such that differentiated T cells derived from the population express recombinant TCRs that enable the cell to recognize and destroy specific types of cancer cells.

Construction of Vector. The segments of DNA which encode the TCR α and β chains are obtained from a T cell clone expressing a TCR of the desired specificity. The genes are then cloned as described below. The genes may be cloned into the same vector, if so only one transduction event need occur; or into separate vectors with the concomitant need for separate transduction events to take place.

The DNA encoding the receptor is inserted into the LXSN bicistronic retroviral vector as described by Miller and Rosman, under the transcriptional control of the LTR from the Moloney Murine Leukemia Virus, along with the neomycin phosphotransferase gene under the transcriptional control of the SV-40 early region promoter. Transcription of the genes is under the control of an endogenous T cell promoter. Using this system, is possible to package TCR α and β transcripts into retroviral particles and transduce hHSC's, selecting for G418 resistance.

Genetic modification of $CD34^+Thy\text{-}1^+$ Population

Bone marrow or mobilized peripheral blood is removed from the human host using standard techniques. $CD34^+Thy\text{-}1^+$ cell populations are isolated and expanded using the

techniques outlined above. The infection is performed by introducing approximately 10^4 $CD34^+Thy\text{-}1^+$ cells and 1 x 10^6 CFU of the virus in 1 ml of long-term culture medium ("LTCM") comprising 10 ng/ml of LIF, IL-3, IL-6, and GM-CSF (or additional cytokines). The cell mixture is maintained for at least 24 hours, and the medium diluted to 10 ml with LTCM and 100 μl of the medium introduced into wells in which confluent layers of the AC6.21 stromal line is present. The cells from 5-15 wells, each of the cells with and without virus, are then introduced into a methylcellulose culture and maintained for 2 weeks. At the end of this time, all cells are collected from each methylcellulose culture and analyzed using Southern and Northern analysis to confirm successful gene insertion and transcriptional expression.

Long-Term T-cell Reconstruction. Cells are then studied for their ability to provide long-term T cell reconstruction as described above.

Cell Therapy. During modification of the cells, the host is treated with high-dose chemotherapy which may substantially or completely ablate native hematopoietic capability. After completion of the treatment of the host, the modified stem cells are then restored to the host to provide for the recognition and destruction of specific types of cancer cells. In addition, unmodified HSC may be administered simultaneously with the modified HSC to reconstitute multilineage hematopoietic function. If necessary, the process is repeated to ensure the substantial absence of the tumor cells and the substantial population of the modified stem cells. T cells derived from gene-modified hHSC that mature in a patient after myelosuppressive or myeloablative regimen are endowed with the specificity to which the binding region of the recombinant TCR has specificity. Further expansion (or activation of) the relevant T cell population could be achieved by the systemic

administration to the patient of appropriate recombinant cytokines (e.g. IL-2, IL-7).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

WHAT IS CLAIMED IS:

1. A composition comprising a human hematopoietic stem cell containing a recombinant DNA molecule encoding a polypeptide comprised of a signal transducing region and an antigenic specificity region.

2. The composition according to claim 1, wherein the recombinant DNA molecule encodes a T cell receptor.

3. The composition according to Claim 2, wherein the T cell receptor comprises α and β chains.

4. The composition according to Claim 2, wherein recombinant DNA further comprises a marker gene.

5. The composition according to Claim 4 wherein the marker gene encodes G418 resistance or stable cell surface protein.

6. A human hematopoietic cell descended from the composition according to Claim 1.

7. A human hematopoietic cell descended from the composition according to Claim 2.

8. The composition according to claim 1 wherein the recombinant DNA molecule encodes a chimeric protein comprising a functional signal transduction region of a T cell specific receptor and an antigen binding site.

9. The composition according to claim 8 wherein the antigen binding site comprises a single chain recombinant molecule.

10. The composition according to claim 8 wherein the antigen binding site comprises the V_H and V_L regions.

11. The composition according to claim 8 wherein the antigen binding site is derived from a monoclonal antibody.

12. The composition according to claim 8 wherein the T cell specific receptor is the Fc receptor γ.

13. The composition according to claim 8 wherein the T cell specific receptor is the TCR ζ.

14. The composition according to claim 8 wherein the T cell specific receptor is at least one of the IL-2 receptor alpha, beta or gamma chains.

15. The composition according to claim 8 wherein said antigen binding site binds to a coat protein of HIV-1.

16. The composition according to claim 8 wherein said antigen binding site is derived from the monoclonal antibody specific to a tumor-associated, cell surface antigen.

17. The composition according to claim 8 wherein said chimeric protein comprises the variable regions of an antibody for a HIV-1 coat protein and the γ chain of the Fc receptor.

18. The composition according to claim 8 wherein said chimeric protein comprises the variable regions of the antibody to a tumor associated, cell surface antigen, and the γ chain of the Fc receptor.

19. A human hematopoietic cell descended from the composition according to Claim 8.

20. A method for immunotherapy comprising the steps of:

withdrawing hematopoietic cells from a human host;

substantially purifying stem cells from the hematopoietic cells;

genetically engineering the stem cells with the recombinant DNA of Claim 1; and

returning the modified cells to the host.

21. The method according to claim 20 wherein cytokines are administered to the purified stem cells.

22. The method according to claim 20 further comprising the step of expanding the stem cells ex vivo.

23. A method for immunotherapy comprising the steps of:

withdrawing hematopoietic cells from a human host;

substantially purifying stem cells from the hematopoietic cells;

genetically engineering the stem cells with the recombinant DNA of Claim 2; and

returning the modified cells to the host.

24. The method according to claim 23 wherein cytokines are administered to the purified stem cells.

25. The method according to claim 23 further comprising the step of expanding the stem cells ex vivo;

26. The method according to Claim 23 wherein the T cell receptor comprises α and β chains.

27. The method according to Claim 23, wherein recombinant DNA further comprises a marker gene.

28. The method according to Claim 27 wherein the marker gene encodes G418 resistance.

29. The method according to Claim 27 wherein the marker gene encodes a stable cell surface antigen.

30. A method for immunotherapy comprising the steps of:

withdrawing hematopoietic cells from a human host;

substantially purifying stem cells from the hematopoietic cells;

genetically engineering the stem cells with the recombinant DNA of Claim 8; and

returning the modified cells to the host.

31. The method according to claim 30 wherein cytokines are administered to the purified stem cells.

32. The method according to claim 30 further comprising the step of expanding the stem cells ex vivo.

33. The method according to Claim 30 wherein the antigen binding site comprises a single chain recombinant molecule.

34. The method according to claim 30 wherein the antigen binding site comprises the V_H and V_L regions.

35. The method according to claim 30 wherein the antigen binding site is derived from a monoclonal antibody.

36. The method according to claim 30 wherein the T cell specific receptor is the Fc receptor γ.

37. The method according to claim 30 wherein the T cell specific receptor is the TCR ζ.

38. The method according to claim 30 wherein the T cell specific receptor is at least one of the IL-2 receptor alpha, beta or gamma chains.

39. The method according to claim 30 wherein said antigen binding site binds to a coat protein of HIV-1.

40. The method according to claim 30 wherein said antigen binding site is derived from the monoclonal antibody against a tumor-associated, cell surface antigen.

41. The method according to claim 30 wherein said chimeric protein comprises the variable regions of an antibody for a HIV-1 coat protein and the γ chain of the Fc receptor.

42. The composition according to claim 30 wherein said chimeric protein comprises the variable regions of the antibody to a tumor-associated, cell surface antigen, and the γ chain of the Fc receptor.

43. A method for immunotherapy comprising the steps of:

withdrawing hematopoietic cells from a human host;

substantially purifying lymphoid progenitor cells from the hematopoietic cells;

genetically engineering the lymphoid progenitor cells with the recombinant DNA of Claim 1; and

returning the modified cells to the host.

44. The method according to claim 43 wherein cytokines are administered to the purified lymphoid progenitor cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10033

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/93.21; 435/172.1, 172.3, 240.2, 240.21, 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 435/172.1, 172.3, 240.2, 240.21, 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: APS, CA, MEDLINE, BIOSIS
Search Terms: Mule?/au; chimer?, T (w) cell?; hematopo?; gene; therapy; hiv; immunodefic?; tcr; recept?; env?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FASEB JOURNAL, Volume 6, issued December 1992, G. Gross et al., "Endowing T cells with antibody specificity using chimeric T cell receptors", pages 3370-3378, see entire article.	1-44
Y	CANCER, Volume 67, No. 10, Supplement, issued 15 May 1991, A.W. Nienhuis et al., "Gene transfer into hematopoietic stem cells", pages 2700-2704, see entire article.	1-44
Y	EXPERIMENTAL HEMATOLOGY, Volume 18, issued 1990, E. Beutler et al., "Gene transfer in the treatment of hematologic disease", pages 857-860, see entire article.	1-44

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 NOVEMBER 1994	DEC 27 1994
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 260, issued 28 May 1993, M.I. Johnston et al., "Present Status and Future Prospects for HIV therapies", pages 1286-1293, see entire article.	1-44
Y	SCIENCE, Volume 260, issued 14 May 1993, R.C. Mulligan, "The basic science of gene therapy", pages 926-931, see entire article.	1-44
Y	HUMAN GENE THERAPY, Volume 2, issued 1991, K.W. Culver et al., "Lymphocyte gene therapy", pages 107-109, see entire article.	1-44
Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 208, issued 1992, K. Roemer et al., "Concepts and Strategies for human gene therapy", pages 211-225, see entire article.	1-44
Y	B.N. Fields et al., "Fundamental Virology", published 1991 by Raven Press, Ltd., (N.Y.), pages 267-290 and 709-723, see entire citation.	1-44

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 43/04, 63/00; A61K 31/70, 38/00; C12N 5/22, 15/00, 15/06, 15/09